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<p>Beta-adrenergic receptor concentration and adenylate cyclase activity change with macrophage activation. Resident (R) and thioglycollate (TG) - stimulated macrophages (Mφ) have comparable concentrations of beta-adrenergic receptors (2136 ± 143 and 2110 ± 327 sites/cell respectively), while Bacillus Calmette Guerin (BCG)-activated macrophages have 62% fewer beta-adrenergic receptors (808 ± 190 sites/cell). The increase in adenylate cyclase activity in TG-Mφ as compared to resident-Mφ results in increased sensitivity to catecholamines.</p> <p>FcR-mediated phagocytosis is dependent on FcR concentration, internalization and intra-cellular digestion of receptor-bound material. Catecholamines increase the concentration of FcR which in turn increases the availability of immune complexes for phagocytosis by the cell. Catecholamines also decrease internalization and/or digestion of FcR-bound immune complexes. Thus, the net effect of catecholamines on total phagocytosis is the consequence of the balance of independent</p>					
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effects of catecholamines on FcR concentration and the efficiency of internalization and degradation of bound material. TG-M ϕ , as described above, are the most sensitive to the effects of catecholamines which is confirmed functionally. TG-M ϕ demonstrate the greatest increase in FcR concentration and inhibition of internalization and digestion, resulting in no net change in phagocytosis. In R-M ϕ and BCG-M ϕ the effects on internalization and digestion override the modest increase in FcR concentration; thus causing a significant decrease in total phagocytosis.

These observations show that the influence of catecholamines on M ϕ phagocytic function are complex and depend on the state of macrophage activation, regulation of beta-adrenergic receptor concentration and adenylate cyclase activity, Fc receptor concentration, and activity of post-FcR functions including rates of internalization and digestion of FcR-bound material. Additional studies are needed to further understand the mechanisms involved in catecholamine-mediated regulation of macrophage phagocytosis.

In separate studies it was found that resident and activated macrophages do not express alpha₂-adrenergic receptors.

Two macrophage cell-lines which express Fc receptor have been assessed for the presence of beta-adrenergic receptors. It was found that one cell-line did and the other did not express beta-adrenergic receptors. These cell-lines provide a model system in which to investigate the mechanism of catecholamine-mediated regulation of macrophage function.

Studies are in progress on the assessment of beta-adrenergic receptor function and activity of M ϕ harvested from stressed animals.

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I. INTRODUCTION

Fc-receptor-mediated phagocytosis of soluble immune complexes and antibody-coated bacteria are important functions of macrophages. Phagocytosis of antigens by macrophages plays a role in antigen clearance, as well as antigen processing for presentation to lymphocytes which initiates cell-mediated and humoral immune responses. The purpose of this project is to study catecholamine-mediated alterations in macrophage phagocytic function.

II. PROGRESS REPORT

a. Regulation of beta-adrenergic receptor expression and adenylate cyclase activity following macrophage activation.

Beta-adrenergic receptor concentration and adenylate cyclase activity were determined on resident (R-M ϕ), thioglycollate-stimulated (TG-M ϕ) and Bacillus Calmette Guerin-activated (BCG-M ϕ) rat peritoneal macrophages. Beta-adrenergic receptor concentration as determined by 125 I-iodocyanopindolol binding was: R-M ϕ , 2136 ± 143 ; TG-M ϕ , 2110 ± 327 , and for BCG-M ϕ , 808 ± 190 sites/cell (mean \pm SEM; $p < 0.01$, ANOVA BCG-M $\phi <$ R-M ϕ and TG-M ϕ). NaF-stimulated adenylate cyclase activity was: R-M ϕ , 35.4 ± 6.6 ; TG-M ϕ , 72.7 ± 13.7 , and for BCG-M ϕ , 32.4 ± 11.9 picomoles cAMP/ 10^6 cells/min (mean \pm SD; $p < 0.01$ ANOVA, TG-M $\phi >$ R-M ϕ and BCG-M ϕ), while isoproterenol-stimulated activity was: 16.0 ± 1.8 , 31.2 ± 3.8 , 15.0 ± 1.3 , respectively (mean \pm SD, $p < 0.05$, ANOVA TG-M $\phi >$ R-M ϕ and BCG-M ϕ). These data suggest that beta-adrenergic receptors and adenylate cyclase activity are differentially and separately regulated during macrophage activation. Adenylate cyclase activity is rate limiting, since resident and BCG cells reflect equal isoproterenol-stimulated activity in the face of different receptor concentrations. The data also demonstrate that TG cells are most responsive to beta-adrenergic stimulation.

The manuscript describing these studies is attached.

b. Catecholamine-mediated regulation of Fc-receptor concentration and phagocytic activity of rat peritoneal macrophages.

The influence of catecholamines of Fc receptor (FcR) concentration was measured at various stages of macrophage activation. Resident, TG-stimulated and BCG-activated peritoneal M ϕ were harvested and incubated with radiolabeled heat aggregated rat IgG (ARG). FcR concentration was determined by Scatchard analysis of binding data. As the model immune complexes contain multiple Fc regions per aggregate and thus can participate in multi-site attachment, these measures of FcR concentration are only meaningful as a relative, but not an absolute, measure of the changes in FcR concentration that are associated with metaproterenol treatment.

EFFECT OF METAPROTERENOL ON M ϕ FcR DENSITY

		Metaproterenol (M)				
		0	10^{-8}	10^{-6}	10^{-4}	10^{-2}
Macrophages (n)						
Resident	3	6.0	6.8	8.3	9.5	5.8
TG	3	7.5	8.0	9.7	12.2	7.4
BCG	3	3.4	3.5	3.0	3.4	3.9

FcR density is expressed as the binding maximum ($\times 10^{-12}$ M). These results demonstrate that FcR density on TG-M ϕ > Resident-M ϕ > BCG-M ϕ . In Resident and TG-M ϕ , there is a dose-dependent increase in FcR density with the addition of metaproterenol up to 10^{-4} M. The addition of high concentrations of metaproterenol (10^{-2} M) is associated with a reduction in FcR density to levels comparable to untreated M ϕ . BCG-M ϕ are not responsive to metaproterenol. Changes in FcR density were not altered by the addition of cycloheximide (2 μ g/ml), suggesting that the effect of metaproterenol does not require new protein synthesis. Prostaglandin E₂ is released from M ϕ following phagocytosis of immune complexes and like metaproterenol it activates adenylate cyclase. Binding studies were performed at 4° C after pre-treatment with metaproterenol at 37° C, thus it is unlikely that internalization of bound material lead to release of prostaglandins. However, to eliminate a possible role of prostaglandin-mediated increase in cyclic AMP, cells were pre-treated with indomethacin (10^{-6} M). Indomethacin pre-treatment did not alter the results observed with metaproterenol treatment. These studies indicate that catecholamine treatment of M ϕ is associated with a dose-dependent increase in FcR concentration on resident and TG-M ϕ . Current studies are in progress to define the effect of metaproterenol or subclasses of FcR. Stimulation of an increase in expression of a non-phagocytic FcR or one that predominantly influences antibody dependent cell-mediated cytotoxicity would have important implications in understanding the effects of catecholamines on FcR-mediated functions.

The influence of catecholamines on FcR-mediated phagocytosis of model immune complexes was studied by incubation of R-M ϕ , TG-M ϕ and BCG-M ϕ with radiolabeled ARG at 37° C and measurement of binding, internalization and digestion of ARG. In resident (21 vs 13%) and BCG (19 vs 11%) M ϕ , 10^{-4} M metaproterenol induces a decrease in the total amount of ARG digested over 2 hours of incubation. In contrast to these observations, metaproterenol treatment of TG-M ϕ is associated with no change in net digestion of ARG (45 vs 43%). As metaproterenol treatment induces a doubling of the amount of ARG bound to TG-M ϕ (as shown in the Table above), and a decrease in the fraction of bound ARG that are internalized, there is no net change in the amount digested. In resident and BCG-M ϕ , the effect of metaproterenol to decrease the rate of internalization/digestion of FcR-bound material is greater than its effect on binding; thus, the net effect is a decrease in digestion. These observations demonstrate that catecholamines effect more than one step of FcR-mediated phagocytosis. Additional studies are needed to further define the influence of catecholamines on each step of phagocytosis and the major subclasses of FcR for IgG.

The manuscript describing these studies is in preparation. Abrass CK, Abrass IB. Catecholamine-mediated regulation of Fc receptor expression on rat peritoneal macrophages.

c. Alpha-adrenergic receptor expression on rat peritoneal macrophages.

Studies described above demonstrated that rat peritoneal macrophages express beta-adrenergic receptors. However, endogenous catecholamines function as both alpha- and beta-adrenergic agonists. Thus, it seemed possible that should macrophages express both alpha₂- and beta-adrenergic receptors, catecholamine-mediated activation of both receptors might produce complex and even opposite effects. Resident, TG-stimulated and BCG-activated rat peritoneal macrophages were harvested and assayed for alpha-adrenergic receptors using [³H]-yohimbine. The absence of specific binding of [³H]-yohimbine to rat macrophages suggests that they do not express alpha₂-adrenergic receptors.

d. Beta-adrenergic receptor expression on mouse macrophage cell-lines.

Previous studies have evaluated net FcR expression and net phagocytic function of rat peritoneal macrophages. Yet, three major subclasses of FcR for IgG have been characterized on human, mouse and rat macrophages. These FcR differ in their amino acid sequence, ligand binding affinity, trypsin-sensitivity, activation of second messengers, as well as activation of phagocytosis, enzyme release and other effects of FcR-ligation. These characteristics of FcR have been most thoroughly evaluated using mouse macrophage cell-lines. Monoclonal antibodies specific for two of the subclasses of mouse FcR for IgG are available. Thus, utilization of these reagents provides the opportunity to determine any FcR-subclass specific catecholamine-mediated effects on FcR function.

Mouse macrophage-like cell lines (P388D₁ and J744a) were grown in culture and assayed for beta-adrenergic receptor expression using standard methods.

P388D₁ cells do not express beta-adrenergic receptors; however, J744a cells bind 98 fmole [¹²⁵I]-iodocyanopindolol/per mg membrane protein. A macrophage (P388D₁) that expresses FcR and is phagocytically active but lacks beta-adrenergic receptors will provide a useful comparison for the J744a line which expresses both activities. Hybridomas for monoclonal antibodies to FcR II and FcR III (2.4G2 and 3G8 respectively, kindly provided by Dr Jay Unkeless) are being cultured. The monoclonal antibodies will be isolated and used to study catecholamine-mediated effects on each subclass of FcR. Aggregated mouse IgG will be used as described above to assay phagocytic activity. These studies are in progress.

Demonstration of differential expression of β -adrenergic receptors on 2 macrophage cell lines, both of which Fc receptors and phagocytic activity, provides a model system in which to investigate the mechanism of catecholamine-mediated regulation of macrophage function. cDNA probes for the β -adrenergic receptor and transfection technology are available in Dr Itamar Abrass' laboratory and cDNA and oligonucleotide probes for each subclass of FcR are available for such studies. The scope of these studies are beyond the directives and budget of the present proposal but will be fruitful avenues for future work.

e. Preliminary studies of β -adrenergic receptor expression and activity of M ϕ harvested from stressed animals.

As described above, it has been suggested that catecholamine β -sensitivity is restored during chronic stress, but no previous studies have been performed to measure β -adrenergic receptor function at that time. Macrophages were harvested from control and stressed rats. Rats were stressed for four weeks by handling each rat for 2 min per day, 5 days per week. The results in the following table show β -receptor number, antagonist and agonist affinities and basal and NaF- and isoproterenol-stimulated adenylate cyclase activity. Cells from 9 control and 9 stressed animals were collected. Three pools of cells from 3 animals each were studied per group. Although there was some variability in the data and this pilot experiment will need to be repeated, the increased agonist affinity and increased isoproterenol responsiveness observed in M ϕ from stressed animals in the face of similar receptor concentrations and adenylate cyclase activity suggest that stress modifies catecholamine sensitivity. Phagocytic function studies are in progress.

$M\mu$ β -RECEPTORS AND CYCLASE IN CONTROL AND STRESSED RATS

	<u>Control</u>		<u>Stressed</u>	
Receptors (No./cell)	1877	\pm 523	1763	\pm 308
Antagonist K_D (10^{-11} M)	1.83	\pm 0.11	1.61	\pm 0.05
Agonist K_D (10^{-8} M)	4.18	\pm 1.09	2.50	\pm 0.21
Cyclase (pmoles/mg/min)				
Basal	60.53	\pm 0.63	52.11	\pm 3.49
GTP	143.81	\pm 25.88	147.18	\pm 33.20
NaF	340.39	\pm 31.49	339.71	\pm 49.06
Iso	10.20	\pm 4.00	50.87	\pm 6.09

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BETA-ADRENERGIC RECEPTORS DURING MACROPHAGE ACTIVATION

REGULATION OF BETA-ADRENERGIC RECEPTOR EXPRESSION AND ADENYLATE
CYCLASE ACTIVITY DURING MACROPHAGE ACTIVATION¹

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ABSTRACT

Beta-adrenergic receptor concentration and adenylate cyclase activity were determined on resident (R-MØ), thioglycollate-stimulated (TG-MØ) and Bacillus Calmette Guerin-activated (BCG-MØ) rat peritoneal macrophages. Beta-adrenergic receptor concentration as determined by 125 I-iodocyanopindolol binding was: R-MØ 2136 ± 143 , TG-MØ 2110 ± 327 , and for BCG-MØ 808 ± 190 sites/cell (mean \pm SEM; $p < 0.01$, ANOVA BCG-MØ $<$ R-MØ and TG-MØ). NaF-stimulated adenylate cyclase activity was: R-MØ 35.4 ± 6.6 , TG-MØ 72.7 ± 13.7 , and for BCG-MØ 32.4 ± 11.9 picomoles cAMP/mg protein/min (mean \pm SD; $p < 0.01$, ANOVA, TG-MØ $>$ R-MØ and BCG-MØ), while isoproterenol-stimulated activity was: 16.0 ± 1.8 , 31.2 ± 3.8 , 15.0 ± 1.3 , respectively (mean \pm SD, $p < 0.05$, ANOVA TG-MØ $>$ R-MØ and BCG-MØ). These data suggest that beta-adrenergic receptors and adenylate cyclase activity are differentially and separately regulated during macrophage activation. Adenylate cyclase activity is rate limiting, since resident and BCG cells reflect equal isoproterenol-stimulated activity in the face of different receptor concentrations. The data also demonstrate that TG cells are the most responsive to beta-adrenergic stimulation.

INTRODUCTION

Previous studies have demonstrated that catecholamines influence macrophage function. Incubation of macrophages with adrenergic agents results in increased production of cyclic AMP (cAMP) (1-5). Elevated levels of intracellular cAMP are associated with decreased phagocytosis of *Trypanosoma cruzi* (6) and IgG-coated erythrocytes (7,8) by mouse macrophages. Alterations in cAMP concentrations influence the macrophage response to chemotactic (9,10) and migration inhibitory factors (11). Macrophage secretion of elastase (12) and collagenase (13) are enhanced by increasing concentrations of cAMP. In further support of the influence of catecholamines on macrophage function, the beta-adrenergic receptor of the rat peritoneal bacillus Calmette Guerin-activated macrophage was characterized (14). Stimulation of this receptor with metaproterenol resulted in a dose-dependent decrease in the net phagocytosis of soluble, model immune complexes by these macrophages (14). The present studies were undertaken to further investigate the role of catecholamine-mediated regulation of macrophage function during macrophage activation. Beta-adrenergic receptor expression and adenylate cyclase activity were compared in resident, thioglycollate-stimulated and bacillus Calmette Guerin activated rat peritoneal macrophages.

MATERIALS AND METHODS

The following reagents were purchased from the manufacturer: HBSS with and without calcium and magnesium

(Gibco, Grand Island, NY), Percoll (Pharmacia Fine Chemicals, Piscataway, NJ), BSA (Miles Laboratories Inc., Elkhart, ID), bacillus Calmette Guerin (BCG²) vaccine (Glaxo Operations, UK, Ltd., Greenford, England), GF/C filters (Whatman, Clifton, NJ), [¹²⁵I]iodocyanopindolol, specific activity 2200 Ci/mole, and radioimmunoassay kits to measure cAMP (New England Nuclear Corp., Boston, MA), thioglycollate (TG) (Becton Dickinson, Cockeysville, NJ).

Isolation of peritoneal macrophages: Sprague-Dawley rats (200 gm) were purchased from Hilltop Laboratories (Scottsdale, PA). Peritoneal macrophages (M ϕ) were isolated by peritoneal lavage with HBSS lacking divalent cations containing 100 mg/l EDTA (15) after no pre-treatment (resident (R) cells), 3 days following intraperitoneal injection of 3 ml of 3% thioglycollate (TG cells) and 7 days following intraperitoneal injection of 1 vial (8×10^4 viable organisms) of BCG (BCG cells). Cells were sedimented by centrifugation ($200 \times g$ for 10 min), resuspended at 1 to 2×10^7 cells/ml in fresh buffer, layered onto a preformed continuous Percoll gradient, and centrifuged at $2,200 \times g$ for 15 min at 4°C (16, 17). Gradients were generated by centrifugation ($26,000 \times g$, 4°C , 30 min.) of a 50% mixture of Percoll and HBSS lacking divalent cations. The macrophage layer was harvested with a pipet, and the cells were washed free of Percoll with excess buffer. Cells were resuspended in the desired buffer as described below. Cell counts were performed using a model ZF Coulter Counter (Coulter Electronics, Miami, FL). Macrophage

purity was monitored by staining for non-specific esterase (18). Cell viability was monitored by trypan blue exclusion prior to and at the end of study. By these criteria >85% of the cells were viable macrophages.

Catecholamine binding studies and adenylate cyclase activity: These studies were performed by previously published methods in which lymphocytes were studied (19-21) and for characterization of the macrophage beta-adrenergic receptor (14). Membranes were prepared by hypotonic lysis of macrophages as previously described (14).

Binding assays: β -adrenergic receptors were quantified by Scatchard analysis of [125 I]iodocyanopindolol (125 I-ICYP) binding. Membranes derived from 10^6 macrophages and 125 I-ICYP (5 to 200 pM) were incubated with and without 1 μ M propranolol for 90 min. at 37°C in a total volume of 250 μ l of assay buffer described above. Nonspecific binding determined in the presence of 1 μ M propranolol was 50% of total 125 I-ICYP binding. Bound was separated from free ligand by vacuum filtration on GF/C disc

Adenylate cyclase: Membranes derived from 10^6 macrophages were incubated in a total volume of 150 μ l of 50 mM HEPES (pH 7.4), 8 mM KCl, 6 mM MgCl, 1 mM ATP, 2.5 mM creatine phosphate, 0.075 mg/ml creatine phosphokinase, and 0.4 mg/ml BSA for 15 min at 37°C. Assays contained either 10 mM NaF, 10^{-4} M GTP, or 10^{-4} M GTP plus 10^{-8} to 10^{-4} M isoproterenol as stimulants. cAMP was quantified by radioimmunoassay (New England Nuclear Corp., Boston, MA.)

Statistical analysis: Multiple groups were compared by one way analysis of variance with subgroup testing by contrast. Scatchard curves were generated from linear regression using least squares.

RESULTS

Characteristics of the peritoneal macrophages: The yields and staining characteristics of each of the types of macrophages are summarized in Table I. Cell yield varied with level of activation, but macrophage purity, as determined by staining for non-specific esterase, was similar in the three groups.

Quantification of the beta-adrenergic receptor: Scatchard analysis of specific binding of ^{125}I -ICYP to macrophages yielded a straight line consistent with a single class of antagonist binding sites. The results of the receptor numbers per cell are summarized in Table II. Resident and TG cells had similar numbers of beta-adrenergic receptors with both having greater than twice the number for BCG cells.

Adenylate cyclase activity: The results of adenylate cyclase activity for each of the macrophage groups are summarized in Table III. Both NaF- and isoproterenol-stimulated adenylate cyclase activities were similar in resident and BCG cells. TG cells had twice the activity of the other cells in response to both stimulants. The concentration of isoproterenol for half-maximal stimulation of adenylate cyclase was similar in the three cell types.

DISCUSSION

Previous studies from this laboratory have demonstrated and characterized the β -adrenergic receptor on BCG-activated rat peritoneal macrophages (14). The BCG-M ϕ was initially chosen over R-M ϕ because of the high yield of cells that could be obtained from BCG-injected rats. It was expected that the higher yield of cells would facilitate the initial characterization of the receptor. The present studies demonstrate that β -adrenergic receptor expression is modulated with activation of the macrophage. The 50% fall in receptor concentration in the BCG-activated cell was unexpected.

Activation of macrophages has previously been demonstrated to be associated with modulation of expression of other cell surface proteins (22-24) and stimulation or suppression of various macrophage functions (25-27). Although expression of the insulin receptor on activated lymphocytes has been shown to be modulated (28), this is the first report of similar modulation of expression of receptors for classical hormones on the macrophage. Stimulation of macrophages by peritoneal injection of thiogylcollate was associated with no significant change in the density of receptors as compared to resident peritoneal macrophages; however, there was a significant rise in adenylate cyclase activity in TG-M ϕ . The concomitant increase in isoproterenol stimulated activity suggests that the β -adrenergic receptor and adenylate cyclase remain coupled. In BCG-M ϕ , receptor concentration was about half of that in R-M ϕ and TG-M ϕ ;

however, adenylate cyclase activity in BCG-M ϕ was similar to that of resident peritoneal cells. Taken together, the data in these three cell types indicate that β -adrenergic receptor concentration and adenylate cyclase activity are independently regulated during M ϕ activation. The higher level of cAMP generation with isoproterenol treatment in TG-M ϕ as compared to R-M ϕ , cells with equal β -adrenergic receptor concentrations, and the equal isoproterenol response between R-M ϕ and BCG-M ϕ , where receptor numbers are altered, indicate that adenylate cyclase activity is rate limiting for catecholamine action in these cells.

Previous studies from this (14) and other laboratories (6) have shown that catecholamine stimulation of and elevated cAMP levels in M ϕ are associated with significant impairment of phagocytosis. Phagocytic activity is modulated during macrophage activation (29) and TG-M ϕ are phagocytically more active than are R- and BCG-M ϕ (30). Demonstration in the present studies that TG-M ϕ are the most responsive to catecholamines suggests that catecholamines may have a more pronounced effect on phagocytosis when M ϕ are specifically activated to perform this function.

Phagocytosis of soluble immune complexes by macrophages is a complex process that includes binding to the M ϕ surface via Fc, C3b or Clq receptors (31), internalization of bound ligand, and intracellular degradation in lysosomes. The influence of changes in catecholamine responsiveness during macrophage activation on each of the steps of phagocytosis still needs to be defined.

These experiments are currently in progress.

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FOOTNOTES

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³ Abbreviations: BCG, bacillus Calmette Guerin; ¹²⁵I-ICYP, [¹²⁵I]-iodocyanopindolol; MØ, macrophage; R, resident; TG, thioglycollate.

TABLE I. Macrophage characteristics

	R-MØ	TG-MØ	BCG-MØ	p value
n	8	7	7	
Cell yield per animal, (X 10 ⁷)	1.66±0.22	4.98±0.23	2.39±0.10	<0.01
Nonspecific esterase (% positive)				
	84.8±2.0	85.4±1.0	85.6±2.4	N.S.
% viable	> 95	> 95	> 95	

The results represent the group mean \pm 1 S D, n represents the number of animals studied.

TABLE II. β -adrenergic receptors on rat peritoneal macrophages.

	n	Receptor sites/cell
R-M ϕ	7	2136 \pm 143
TG-M ϕ	6	2110 \pm 327
BCG-M ϕ	6	808 \pm 190

The results represent the group mean \pm 1 SEM, $p < 0.01$, analysis of variance, for BCG-M ϕ compared to R-M ϕ and TG-M ϕ .

TABLE III. Rat macrophage membrane adenylate cyclase activity

Stimulant	Activity		
	R-MØ	TG-MØ	BCG-MØ
	(pmoles cAMP/min/mg membrane protein)		
NaF	35.41±6.55 (8)	72.71±13.74 (4)	32.36±11.93 (7)*
Isoproterenol	15.96±1.79 (4)	31.17±3.82 (4)	15.04±1.25 (3)+
	(10 ⁻⁶ M)		
	(half-maximal stimulation x 10 ⁻⁷ M)		
Isoproterenol	6.90±6.11 (4)	5.11±2.21 (4)	9.42±7.49 (3)

Iso = isoproterenol. Values for basal cAMP and in the presence of GTP were subtracted from values obtained for the other conditions to determine the specific adenylate cyclase activity for NaF and isoproterenol stimulation. The results are expressed as the group mean ± 1 SD of results obtained from individual experiments (n).

* p < 0.01, analysis of variance, TG-MØ > R-MØ and BCG-MØ.

+ p < 0.05, analysis of variance, TG-MØ > R-MØ and BCG-MØ.